Proc. Nat. Acad. Sci. USA
Vol. 71, No. 7, pp. 2882–2886, July 1974

A Cleavage Map of Bacteriophage φX174 Genome

(restriction endonuclease/DNA fragments/DNA mapping/gel electrophoresis)

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Contributed by Robert L. Sinsheimer, April 10, 1974

ABSTRACT **Restriction endonucleases isolated from *Hemophilus influenzae*, *Hemophilus parainfluenzae*, and *Hemophilus aegyptius* were used to cleave φX174 replicative form DNA into three sets of specific DNA fragments. The order of these fragments in the φX replicative form molecule was determined by (*1*) analysis of partial digest products, (*2*) analysis of overlapping sets of fragments produced by two different restrictive enzymes. On the basis of these results, a detailed physical map of the φX174 genome has been constructed with respect to the cleavage sites of all three enzymes.**

The genome of bacteriophage φX174 is a covalently closed circular molecule consisting of a single strand of about 5500 nucleotides (1). Several bacterial restriction deoxyribonucleases have been described which cleave the double-stranded, circular φX174 replicative form DNA (φX RF) into specific fragments (2–4). These enzymes are particularly useful since they hydrolyze DNA at specific sites. In order to use these specific fragments for the study of the structure and function of φX, it is necessary to order these fragments with respect to the φX genetic map (5). By analysis of partial digestion products and of overlapping sets of fragments, we have ordered the three sets of DNA fragments produced by hydrolysis of φX RF by means of the restriction endonucleases from *H. influenzae*, *H. aegyptius*, and *H. parainfluenzae*.

RESULTS

Molecular Size Estimates of DNA Fragments. The origins and the purification of the *Hemophilus* enzymes have been described (3, 4, 6). We have prepared the above three endonucleases and the phosphocellulose column fractions were used to hydrolyze [*U*-^{32}P]φX RF. The resultant fragments were separated on 4% polyacrylamide gels.

The molecular sizes (the number of base pairs) of the fragments were calculated from their relative mobilities by means of a plot relating log molecular size to R_F in a 4% polyacrylamide gel (P. H. Johnson, personal communication). The known molecular sizes of the *Hin* limit digest products R9 and R10 as reported by Maniatis *et al.* (7) were used to standardize this plot. The sizes of the larger fragments, e.g., P1, P2, were also estimated by summation of the molecular sizes of the products derived from them by degradation with a second enzyme. Table 1 summarizes the molecular size estimates thus obtained of φX RF fragments produced by cleavage with the three *Hemophilus* restriction endonucleases. These values are in good agreement with the relative yield of each fragment from [*U*-^{32}P]DNA.

Order of φX Hpa Fragments by Analysis of Partial Digest Products. The general approach for ordering of the *Hpa* fragments was the separation by either polyacrylamide or agarose gel electrophoresis of ^{32}P-labeled fragments obtained by incomplete digestion of φX RF with the *Hpa* restriction enzyme. Each partial product was subsequently eluted and redigested with excess *Hpa* enzyme to produce the final digestion products, and thereby establish a linkage group.

Several partial digests were prepared, the intermediate products purified (Fig. 1*a*), and then redigested. Several examples of the products of redigestion are presented in Fig. 1*b*. Partial product A, for instance, yielded P1, P3, and P4a upon redigestion; partial B yielded only P1 and P4a; partial F yielded P3 and P4a. Partial C yielded P2 and P7 on redigestion. Partial D was actually the same as partial C. Some products isolated from the preparative gels were, in fact, already limit digestion products. Thus, sample E was actually P2, and sample G, P3; these yielded no other fragments upon redigestion.

Table 2 summarizes the data from all partial products analyzed. The order of fragments P1, P3, P4a, and P5 can be deduced as P5-P1-P4a-P3 from overlapping positions. P4b

TABLE 1. *Molecular size estimates of φX174 RF fragments produced by cleavage with Hemophilus restriction endonucleases*

Hin fragment	Size in base pairs	*Hae* fragments	Size in base pairs	*Hpa* fragments	Size in base pairs
R 1	1000	Z 1	1200	P 1	1600
2	760	2	1050	2	1400
3	670	3	870	3	720
4	510	4	600	4**a*	400
5	400	5	320	*b*	390
6**a*	350	6**a*	290	5	350
b	345	*b*	285	6	215
c	340	7	230	7	185
7**a*	300	8	190		
b	290	9	115		
8	205	10	73		
9	155				
10	80				
Total	5405		5223		5260

* These fragments contain multiple tracts very similar in size; each tract is designated by *a*, *b*, *c*, etc.

Abbreviations: φX RF, double-stranded, circular φX174 replicative form DNA; *Hin*, *Hae*, and *Hpa* are restriction enzymes from *H. influenzae*, *H. aegyptius* and *H. parainfluenzae*, respectively, and produce a set of R, Z, and P fragments upon hydrolysis of φX RF.

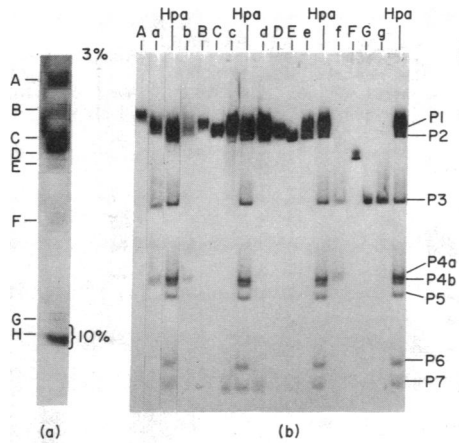

FIG. 1. Examples of partial and complete digests of ^{32}P-labeled φX RF with *Hpa*. (*a*) Separating *Hpa* partials. 10 μg of [^{32}P]φX RF (10^5 cpm/μg) were incubated at 37° for 30 min with 5 μl of the *Hpa* enzyme in a volume of 0.15 ml, containing 7 mM each of Tris·HCl (pH 7.4), $MgCl_2$ and β-mercaptoethanol, and 20 mM of NaCl. Reaction was stopped by addition of ethylenediaminetetraacetate to 0.01 M. Before electrophoresis, the digestion mixture was adjusted to 1% sodium dodecyl sulfate and 15% sucrose. Partials A-G were separated on a 3% (bottom 1 cm is 10%) vertical slab gel (14 × 12 × 0.25 cm) at 45 V for 16 hr. The electrophoresis buffer consists of 0.04 M Tris·HCl, 0.02 M sodium acetate, 1 mM ethylenediaminetetraacetate, 0.1% sodium dodecylsulfate (pH 7.8). The gel chamber used was that described by Studier (8). The DNA bands were located by autoradiography of the wet gel. DNA was eluted from gel segments by two successive incubations in 1 ml of 0.1 × the concentration of SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 48 hr at room temperature. The DNA was then precipitated with ethanol and redissolved in 40 λ of 0.005 M Tris·HCl (pH = 7.4). (*b*) Redigestion of partial digests *A–G*. Complete digests were obtained by incubating 0.2 μg of DNA with 5 μl of enzyme at 37° for 2 hr in the standard buffer described in (*a*). The partial product (*A*, *B*, etc.) and the redigested partial product (*a*, *b*, etc.) were electrophoresed on a 4% gel (14 × 12 × 0.12 cm) along with a complete *Hpa* digest (*Hpa*). Electrophoresis was carried out at 50 V for 6 hr. The gel was dried under vacuum at 100° by the method of Maizel (9), and was then placed in direct contact with Kodak RP54 x-ray medical film for overnight exposure.

was invariably one of the earliest limit products produced and was not obtained by the redigestion of partials.

Redigestion of Hin Fragments with Hpa Restriction Enzyme. To resolve the remaining ambiguity in the order of *Hpa* fragments, i.e., the order of P2, P4b, P6, and P7, as well as to order the *Hin* fragments, we have digested terminal digest *Hin* fragments with the *Hpa* restriction enzyme.

Fig. 2*a* illustrates the results of a simultaneous digestion (to completion) of [^{32}P]φX RF by the *Hin* and *Hpa* restriction enzymes after analysis on 4% polyacrylamide gels. The bands corresponding to R2 and R7a disappeared in the double digest. When the yield of radioactivity of the double digestion products was quantitated, one of the fragments in the R6 band (presumably R6a as judged from the slab gel) was also missing.

On isolation of individual *Hin* fragments and digestion of these with *Hpa*, as shown in Fig. 2*b*, R1, R3, R4, R5, R7b, R8, and R9 were uncleaved. Fragment R2 yielded P5 and P6 and a unique piece (U195) as indicated by *arrow*. This latter piece also derived uniquely from P2, when the reciprocal digestions

TABLE 2. *Redigestion of partial digestion products with Hemophilus parainfluenzae (Hpa) restriction endonuclease*

Partial	*Hpa* products	φX fragment
A	1, 3, 4*a*, 5	5 1 4*a* 3
B	1, 3, 4*a*	1 4*a* 3
C	1, 4*a*	1 4*a*
D	3, 4*a*	4*a* 3
E	2, 7	(7)2
F	2, 7, 6	(7)2(6)
G	1, 4*a*, 5	5 1 4*a*

were made (Table 3). This result led us to conclude that P5, P6, and P2 are linked. Combining these data with those in Table 2, the (circular) order of the *Hpa* fragments was resolved (Fig. 3*a*).

When a mixture of R5, R6, and R7 was digested with the *Hpa* restriction enzymes, R5, two of the R6 components and R7b were not cleaved. However, at least three small fragments of size 220, 185, and 145 base pairs (indicated by *arrows* in Fig. 2*b*) were produced from R6 and R7a. By separate digestion of R6 and R7 with the *Hpa* enzyme, it can be shown that the R6 component yields P7 (185 base pairs) and the unique 145 base pair piece; R7a yields the unique piece of 220 base pairs and a small fragment of 80 pairs.

Redigestion of Hpa Fragments by Hin. To further order the *Hin* fragments, we have determined which *Hin* fragments

TABLE 3. *Redigestion of Hpa fragments by Hin and Hae*

Hpa fragment	Size*	Products with *Hin*†	Product size*	Products with *Hae*†	Product size*
P1	~1600	R3	670	Z6 *a*	290
		R4	510	Z6 *b*	285
		R6	340	ΔZ2	850
		R8	205	Z9	115
			1725		1540
P2	~1400	R1	1000	ΔZ1	1000
		R9	155	ΔZ4	500
		ΔR2	195		1500
			1350		
P3	720	R6	345	ΔZ3	450
		R7 *b*	290	Z7	230
			635		680
P4 *a*	400	R5	400	ΔZ3	400‡
P4 *b*	390	ΔR7 *a*	220	ΔZ5 (?)	255
		ΔR6	145	ΔZ8	120
			365		375
P5	350	ΔR2	350‡	ΔZ2	245
				ΔZ1	110
					355
P6	215	ΔR2	215‡	ΔZ1	215‡
P7	185	ΔR6	185‡	Cleaved to <100	

* Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

† A part of a fragment is denoted by Δ, e.g., ΔR2 means part of fragment R2.

‡ These fragments were uncleaved.

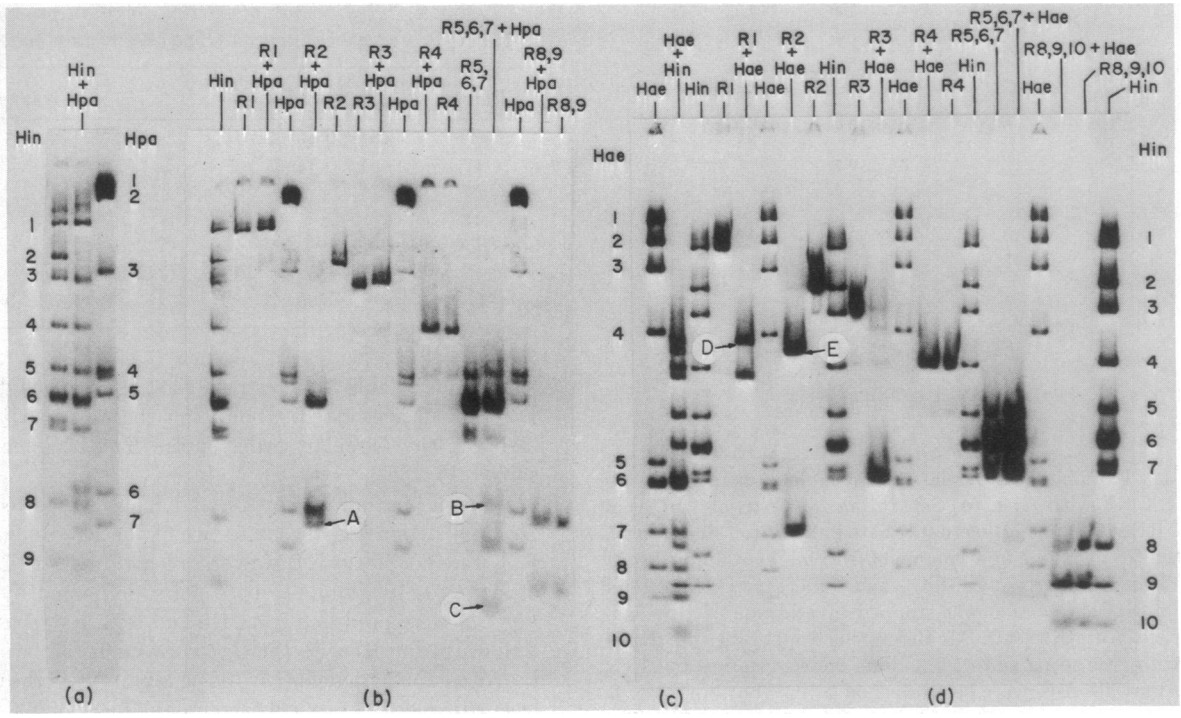

FIG. 2. Digestion of *Hin* fragments with *Hpa* and *Hae*. (*a*) A *Hin* digest is at the *left* and a *Hpa* digest is at the *right*. The *middle column* is a digest produced by simultaneous digestion with *Hpa* and *Hin* enzymes. Conditions for *Hin* digestions were the same as in *Fig. 1b* except the final NaCl concentration was 50 mM. Electrophoresis was carried out on a 5% gel at 40 V for 9 hr. (*b*) Digestion of isolated *Hin* fragments with *Hpa*. Individual *Hin* fragments were recovered by continuous electro-elution from 2.5% agarose cylindrical gels. Details of the method have been described (10). The undigested fragments (R1, etc.) and the redigested fragments (R1 + *Hpa*) were electrophoresed with complete digest markers (*Hin*) and (*Hpa*) as in (*a*). The *arrows* designate the new unique fragments, *A* = U195, *B* = U220, *C* = U145. (*c*) On the *left* is a complete *Hae* digest of ϕX RF. Conditions for *Hae* digestions were the same as in *Fig. 1b*. The *right* is a *Hin* digest. The *middle column* is a digest produced by simultaneous digestion with both enzymes. Electrophoresis was carried out on a 4% gel (bottom 1 cm is 10% gel) at 50 V for 6.5 hr. (*d*) Redigestion of individual *Hin* fragments (recovered from electroelution as in *b*) with *Hae*. Electrophoresis conditions were the same as in *c*. *D* = U590, *E* = U540.

could be derived from the individual *Hpa* fragments. The results of such digestions are in Table 3.

P1, when redigested with the *Hin* restriction enzyme, yields R3, R4, one component of R6 and R8. P2 yields R1, R9, and the same unique fragment (U195) which is derived from R2 when digested with *Hpa* (Fig. 2*b*). P3 yields one component of R6 and R7b; P4b yields two new unique fragments identical with those derived upon digestion of R6 plus R7a by *Hpa*.

For some fragments, e.g., P3, the sum of product molecular size is slightly less than its molecular size estimated from relative mobility. It is possible that an additional small fragment (less than 100 base pairs) was derived upon redigestion but was electrophoresed out of the gel and thus unaccounted for.

A unique situation arose in the relationship of P4a and R5, which have identical electrophoretic mobilities on polyacrylamide gels. Neither P4a or R5 are cleaved by the other enzyme; however, only one fragment with the size of R5 (or P4a) was obtained from a simultaneous *Hin* and *Hpa* digestion. This led us to conclude that P4a and R5 are identical.

Combining the above results, a partial order of the R fragments can be derived as shown in Fig. 3*b*.

Redigestion of Hae Fragments with Hpa and Hin. The restriction enzyme from *H. aegyptius* cleaves ϕX RF into 11 specific fragments; Z6 consists of two fragments of similar size. One approach to resolve the linear order of the four *Hin* fragments derived from P1, the order of R7b and R6, and the order of R1 and R9, is to isolate individual *Hae* fragments and redigest them with *Hpa* and *Hin* restriction enzymes.

When ^{32}P-labeled ϕX RF is simultaneously digested with the *Hae* and *Hpa* restriction enzymes and the resultant digestion products analyzed, P4a, P6, Z6a, Z6b, Z7, Z9, and eight additional major unique fragments (U1000, U850, U500, U450, U255, U245, U120, and U110) can be identified.

Fig. 2*c* illustrates the result of a double digestion of ϕX RF with the *Hin* and *Hae* restriction enzymes. It is evident that R1, R2, R3, as well as Z1, Z2, Z3, Z4, and Z5 were cleaved.

Individual *Hae* fragments were redigested with either the *Hpa* or *Hin* restriction enzymes (Table 4). The identity of unique fragments was determined by digestion of individual *Hpa* and *Hin* fragments with the *Hae* restriction enzyme and analysis of overlapping sets of fragments. Additional linkage groups of *Hin* fragments are derived from redigestion of large *Hae* fragments; e.g., from Z1 it can be concluded that R1, R9, and R2 are linked, in that order.

It is also possible to deduce the order of *Hae* fragments from redigestion data of individual *Hpa* fragments with the *Hae* restriction enzyme (Table 3) (except for Z10 which was electrophoresed out of the gel). The relationship of the *Hpa* and *Hae* fragments is illustrated in Fig. 3*c*.

To confirm the redigestion data, individual *Hin* fragments were digested with the *Hae* restriction enzyme (Fig. 2*d*). The reciprocal digestion results are in agreement with each other. For instance, Z1 was cleaved by the *Hin* restriction enzyme into two large fragments (U590 and U540) and R9 (Table 4). Fig. 2*d* illustrates that the (U590) fragment is derived from R1, the U540 piece from R2, and R9 is uncleaved.

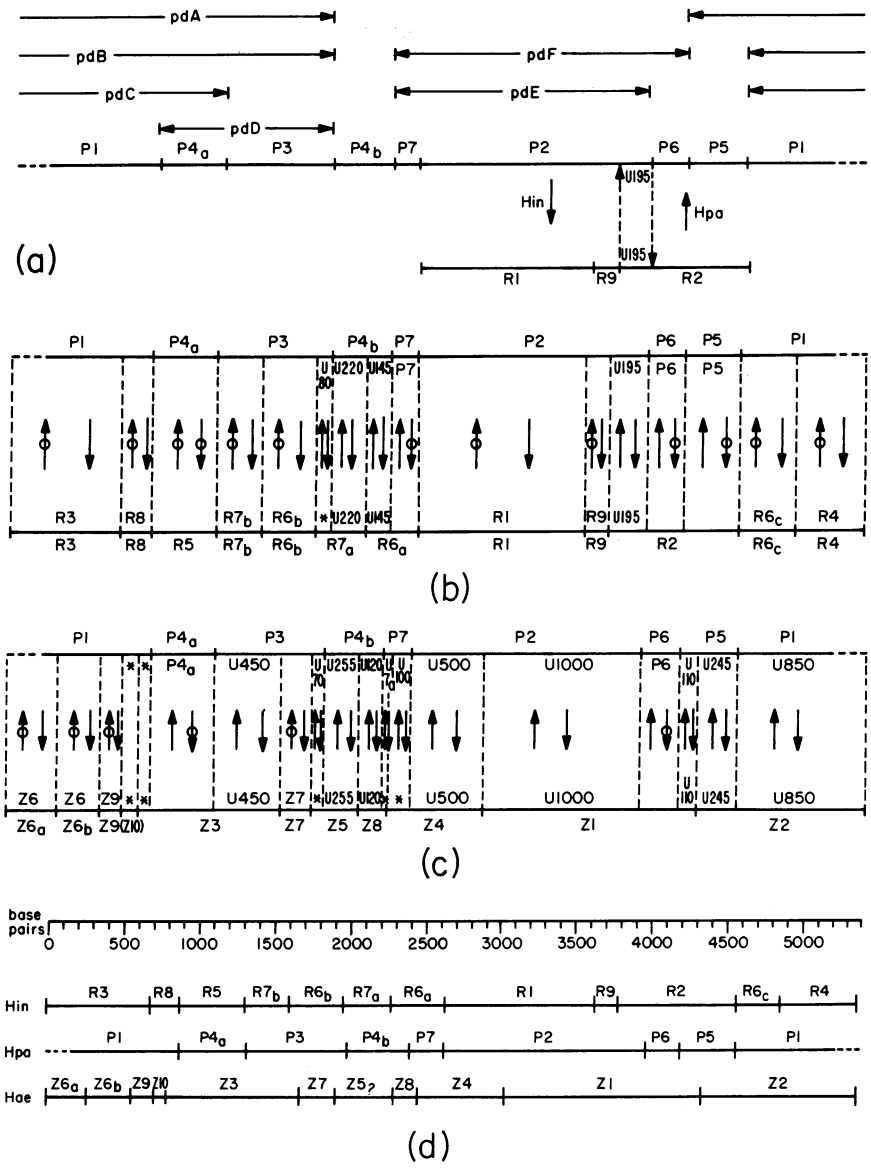

FIG. 3. Derivations of the *Hpa*, *Hin*, and *Hae* cleavage maps. (*a*) Derivation of the *Hpa* cleavage map. *pd*, partial digest fragment (*Hpa*), from *Table 2; P*, complete digest fragment (*Hpa*); *R*, complete digest fragment (*Hin*); *U195*, unique fragment of 195 base pairs from double digest. (*b*) Relationship between *Hpa* and *Hin* fragments. ↑, *Hin* fragments redigested with the *Hpa* enzyme; ↓, *Hpa* fragment redigested with the *Hin* enzyme; ϕ, uncleaved; *, expected small fragment, undetected, presumably electrophoresed out of gel. (*c*) Relationship between *Hpa* and *Hae* fragments. ↑, *Hae* fragment redigested with the *Hpa* enzyme; ↓, *Hpa* fragment redigested with the *Hin* enzyme. (*d*) A cleavage map of the ϕX174 genome with the *Hin*, *Hpa*, and *Hae* restriction enzymes.

We can summarize all data in a single cleavage map of the ϕX genome, which incorporates the various cleavage sites and molecular size estimates of the fragments (Fig. 3*d*).

The assignment of several fragments, e.g., R6c, R4, R8, Z5, Z9, and Z10 deserves comment. From our redigestion data alone, the position of R6c and R4 can be interchanged and R8 can be on either side of R3. However, a partial order of *Hin* fragments has been determined by Johnson and Sinsheimer (11) using *Escherichia coli* DNA polymerase I in an extended repair reaction with uniquely gapped late RF II molecules. Those results indicate that R8 is between R3 and R5. The position of Z5 is considered tentative since it was cleaved by both *Hpa* and *Hin*. The assignment was based on the production of an identical unique fragment from P4b and Z5 on redigestion with the other enzyme.

Both P1 and P5, on redigestion with the *Hae* enzyme, yield a fragment very similar in size to Z9. However, R2, from which P5 was derived (Fig. 2*b*), did not yield Z9 when treated with the *Hae* enzyme. On the other hand, R3 was cleaved into at least three fragments by the *Hae* enzyme, namely the two Z6 fragments and a small piece consisting of 57 base pairs. R8, which is adjacent to R3, yielded two bands when digested with the *Hae* enzyme. The sizes of the fragments in these bands are 75 (equivalent to Z10) and 63 base pairs. The larger band (U75) probably consists of two tracts very similar in size. In addition, Z9 was cleaved by the *Hin* restriction enzyme into two fragments, U57 and U63. This could only occur if Z9 and Z10 are located between R3 and R5 as shown in Fig. 3*d*. The sum of the two small fragments derived from R3 (U57) and R8 (U63) on cleavage with the *Hae* enzyme adds up

TABLE 4. *Redigestion of Hae fragments by Hpa and Hin*

Hae fragment	Size*	Products with *Hin*†	Product size*	Products with *Hpa*†	Product size*
Z1	1200	ΔR1	590	ΔP2	1000
		ΔR2	540	P6	215
		R9	155	ΔP5	110
			1285		1325
Z2	1050	R4	510	ΔP5	245
		R6	340	ΔP1	850
		ΔR2	230		1095
			1080		
Z3	870	R5	400	P4 *a*	400
		R7 *b*	290	ΔP3	450
		ΔR6	115		850
		Small piece	75		
			880		
Z4	600	ΔR1	480	ΔP2	500
		ΔR6	120	ΔP7	100
			600		600
Z5	320	Δ7 *a* (?)	290	ΔP4 *b*	255
		Small piece	~30	Small piece	70
			320		325
Z6 *a*	290	ΔR3	290‡	ΔP1	290‡
b	285	ΔR3	285‡	ΔP1	285‡
Z7	230	ΔR6	220	ΔP3	230‡
Z8	190	ΔR6	190‡	ΔP4 *b*	120
				Small piece	70
					190
Z9	115	ΔR3	57	ΔP1	115‡
		ΔR8	63		
			120		
Z10	73	ΔR8	73‡	—	—

* Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

† A part of a fragment is denoted by Δ, e.g., ΔR2 means part of R2.

‡ These fragments were uncleaved.

to the size of Z9. Our assignment of Z9 and Z10 is in agreement with the partial order of Z fragments determined by L. K. Miller (personal communication) using *in vitro* repair synthesis of gapped late RF II molecules.

DISCUSSION

The physical map of φX as shown in Fig. 3*d* is based on cleavage of φX RF by the *Hin*, *Hpa*, and *Hae* restriction endonucleases. The *Hpa* enzyme we used in our experiments hydrolyzed SV40 into four specific fragments as reported by Sack and Nathans (12). It was reported by Sharp *et al.* (13) that the *Hpa* restriction enzyme contained two enzymes, *Hpa* I and II.

We have, in addition, confirmed the physical order of the *Hpa* fragments by using two individual *Hpa* fragments (P1 and P2) as specific primers for *in vitro* DNA synthesis after annealing the complementary strand of the fragments to φX viral DNA (unpublished results). The nucleotides incorporated were pulse labeled with [^{32}P]dXTP and chased with excess of cold triphosphates. The completed double-stranded molecules were then digested with the *Hpa* enzyme. A gradient of labeling was found such that the *Hpa* fragment adjacent to the priming fragment, e.g., P5 in the case of P1 as primer contained the most ^{32}P-label. This technique will be useful for determining the location of small fragments.

In a partial restriction enzyme fragment map for φX reported by Chen *et al.* (14), R2 was positioned within the Z1 fragment. However, our redigestion data indicated that R2 was cleaved by the *Hae* restriction enzyme into two pieces. This result is consistent with the double digestion data described earlier by Middleton *et al.* (2).

Note Added in Proof. The results obtained by the use of R fragments as specific primers for *in vitro* DNA synthesis have led to the conclusions that R6c is between R2 and R4, that R8 is between R3 and R5, and that R10 is between R9 and R2.

We thank Dr. C. Hutchinson, III for sending us the *H. aegyptius* strain, Dr. P. H. Johnson for the generous supply of [^{32}P]φX RF, and Ms. Carole Latham for excellent technical assistance. This investigation was supported by the McCallum Fund of the Division of Biology of the California Institute of Technology and by USPHS Grant GM13554.

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